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NEONATAL IMMUNIZATION WITH CPG DNA-BASED MICROPARTICLE FORMULATIONS

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Neonatal immunization with CpG DNA based microparticle formulations

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SUMMARY

The overall objective of this project was to develop slow-release microparticles for delivery of DNA-based vaccine formulations, in particular for bovine herpesvirus-1 (BHV-1), and ultimately for additional pathogens involved in bovine respiratory disease. Both a DNA vaccine, consisting of plasmid encoding protective antigen, and a protein vaccine, containing protective antigen formulated with the immune modulator, CpG ODN, were evaluated. Several types of poly(D,L-lactide-co-glycolide) (PLGA) microparticles were produced to be able to i) coat the microparticles with plasmid DNA or CpG ODN (cationic PLGA particles) or with protein (anionic PLGA microparticles); ii) incorporate protein inside the microparticles (polyphosphazene-PLGA microparticles) and iii) modify microparticles to target cells in the respiratory tract (chitosan-coated polyphosphazene-PLGA microparticles).

All microparticles were characterized based on size, and plasmid DNA, CpG ODN and protein loading efficiency. The plasmid-coated microparticles were further characterized *in vitro* based on transfection efficiency of COS-7 cells and dendritic cells (DCs), as well as *in vivo* for protein expression in injected skin biopsies. The protein-coated or encapsulated microparticles were further characterized based on protein release and uptake by a macrophage cell line. Selected microparticles were then evaluated in mice and/or cattle for their ability to induce humoral and cell-mediated immune responses, and protection from BHV-1.

The plasmid-coated microparticles entered cells in tissue culture, and the plasmid expressed protein. Furthermore, the plasmid-coated microparticles were taken up by murine and bovine antigen DCs *ex vivo*, and protein expression was detected. We also demonstrated that delivery of plasmid-coated microparticles into bovine skin leads to local protein expression; however, the protein expression levels were not increased when compared to delivery of plasmid in saline. Furthermore, the delivery of plasmid to mice on the PLGA microparticles did not result in increased immune responses when compared to plasmid delivery in saline.

Several protein microparticles were promising. A number of PLGA microparticles were compared with respect to efficiency of protein loading, protein release, and size. All of the microparticles had a 1-2.5 μm size, which is optimal for uptake by DCs. One of the protein-coated PLGA microparticles induced prolonged immune responses when delivered subcutaneously. Incorporation of protein in polyphosphazene-PLGA microparticles also resulted in stronger and more balanced immune responses in mice and enhanced immune responses in sheep. Polyphosphazene-PLGA microparticles coated with chitosan, which targets to the mucosal surfaces, induced strong immune responses when delivered intranasally to mice. This is of importance for the ability to deliver antigens mucosally, which is expected to lead to improved protection from respiratory disease. Finally, microparticles proved to be a good vehicle for delivery of the immune modulator CpG ODN, which has strong adjuvant activity, both in mice and in cattle.

In conclusion, we have established microparticle formulations that are promising for delivery of protein and CpG ODN, whereas further research is needed for microparticle formulation of DNA vaccines. Future studies will involve optimizing the protein-CpG ODN microparticle formulations as vaccine candidates against viruses involved in bovine respiratory disease, in particular IBR and BVDV.

INTRODUCTION

Background

Bovine respiratory disease causes significant economic losses to the North American cattle industry, because there are no good prophylactic or therapeutic methods for control of BRD. BHV-1, BVDV, BRSV and PIV3 play a major role in BRD or shipping fever (34). These viruses cause high morbidity and predispose calves to secondary bacterial infections, specifically by *P. haem.* and *H. somnus,* which often leads to mortality. New BRD vaccines continue to be introduced emphasizing the market demand for control of economic losses due to shipping fever. Many of these vaccines could be improved and ideally they would be given earlier, either after birth or at branding, rather than after entry in the feedlot. However, the neonatal immune system is functionally immature, so newborns are easily rendered tolerant to allo-antigens (20, 32) following immunization and they specifically appear to have difficulty mounting a cellular immune response (5), which is critical for successful vaccination against many infections. Consequently, newborns are prone to infections and they develop only a weak immune response to most conventional vaccines (28). Secondly, the transfer of maternal antibodies from the mother to the neonate generally provides short-term protection, but at the same time can prevent the development of effective active immunity (27). Since the levels of maternal antibodies decline at different rates, animals may either be vaccinated too late, which results in a window of susceptibility to disease, or too early, in which case vaccination may be ineffective. Neither modified-live nor killed vaccines are effective in the presence of maternal antibodies (27), and the degree of inhibition is correlated to the maternal antibody levels at the time of immunization.

Recently, we and others have shown that the immune system of vertebrates recognizes unmethylated CpG dinucleotides (CpG motifs) in bacterial DNA as a danger signal. Consequently, CpG motifs stimulate the immune system extremely well and support the development of both antibody and cellular immune responses. CpG motifs can be incorporated into DNA vaccines or into synthetic oligodeoxynucleotides (CpG ODNs) and then used as an adjuvant with protein vaccines. CpG ODNs cause either very little and transient, or no tissue damage. Because of these characteristics, the use of plasmid DNA containing CpG motifs or proteins adjuvanted with CpG ODN is a promising approach to the development of immunity in neonates. A DNA vaccine consists of a bacterial plasmid that contains a gene encoding a protective antigen. When animals are immunized with the plasmid, the protective antigen is produced and presented to the immune system in the same manner as during natural infection. This therefore results in both antibody and cell-mediated responses which is crucial for protection from many diseases (2, 12, 19, 25). Indeed, we and others have already shown that DNA vaccines induce both antibody and cellular responses in neonatal mice, lambs, piglets and primates (3, 6, 10, 13, 33). DNA vaccines are easy to engineer and produce, have a long shelf life and do not need refrigeration. The safety of DNA vaccines has been established in several species including humans (4 , 17). A DNA vaccine for dogs is currently on the market, and a West Nile vaccine for horses is in the final stages of development. CpG ODNs have adjuvant activity not only in mice (8, 26), but also in out-bred species (11). We showed that the addition of CpG ODN to a protective antigen of BHV-1, tgD, results in enhanced and more balanced immune responses in cattle (15, 24) and protection from viral challenge (15). Furthermore, CpG ODNs are capable of enhancing antibody and cellular immune responses against hepatitis B antigen in neonatal mice and BHV-1 tgD in neonatal lambs (1, 7).

Rationale

We demonstrated that immunization with a DNA vaccine encoding BHV-1 tgD (33) or with tgD formulated with CpG ODN induces immune responses in newborn lambs. Moreover, priming with a tgD DNA vaccine, followed by boosting with a tgD protein - CpG ODN formulation induced superior immune responses compared to other vaccination approaches. We also confirmed the efficacy of DNA-based vaccination in newborn calves. These vaccines were delivered with a needle-free device, the Biojector, and were effective even in the presence of maternal antibodies. Ideally, producers wish to vaccinate once. Therefore, the goal of this project is to develop a strategy to deliver the DNA vaccine and the protein vaccine in a single shot vaccination. This means that we need to use a slow or pulsed release microparticle-based delivery system that is compatible with both the DNA vaccine and the protein-CpG ODN vaccine. Then we would be able to incorporate the DNA and protein-CpG ODN vaccine either in the same microparticles or in separate microparticles that can be mixed.

Several compounds could achieve this, including poly(D,L-lactide-co-glycolide) (PLGA), polyphosphazenes (PCPP and PCEP) and particles made from emulsifying wax (EW) (9, 21, 23). We first focused on PLGA because 1) antigens incorporated in PLGA particles induce enhanced immune responses in mice, sheep and cattle (16, 30, 31); 2) formulation of DNA vaccines with PLGA microparticles results in significantly enhanced immune responses (29, 30), 3) CpG ODN can be formulated in PLGA particles; and 4) PLGA particles have been successfully delivered with a Biojector.

Objectives

1) Formulation of plasmid and protein in microparticles and *in vitro* characterization.
2) Assessment of release of plasmid and protein from microparticles in animal tissues.
3) Analysis of distribution of protein produced by microparticle-formulated plasmid in animals.
4) Evaluation of immune responses induced by plasmid encoding protective antigen and protein-CpG ODN in microparticles.

METHODS

Several types of poly(D,L-lactide-co-glycolide) (PLGA) microparticles were produced to be able to i) coat the particles with plasmid DNA or CpG ODN (cationic PLGA particles) or with protein (anionic PLGA microparticles); ii) incorporate protein inside the microparticles (polyphosphazene-PLGA microparticles) and iii) modify microparticles to target cells in the respiratory tract (chitosan-coated polyphosphazene-PLGA microparticles).

All microparticles were characterized based on size and DNA, CpG ODN or protein loading efficiency. The plasmid-coated microparticles were further characterized *in vitro* based on transfection efficiency of COS-7 cells and dendritic cells (DCs), as well as *in vivo* for protein expression in injected skin biopsies (18). The protein-coated or encapsulated microparticles were further characterized based on protein release and uptake by a macrophage cell line.

Selected microparticles were then evaluated in mice and/or cattle for their ability to induce humoral and cell-mediated immune responses, and protection from BHV-1 according to our established protocols (14, 15, 18).

RESULTS

1. Formulation of plasmid and protein in microparticles and *in vitro* characterization.

1.1 Production of plasmids and proteins

Plasmid DNA for immunization was purified from *Escherichia coli* strain DH5α using endotoxin-free anion exchange columns (Qiagen), resuspended at 2.0 mg/ml in sterile endotoxin-free water, and stored at -20 °C. Endotoxin levels in DNA stocks were verified to <0.10 EU/mg DNA (<10 pg/mg DNA), using the *Limulus* amoebocyte lysate QLC-1000 kit. Plasmids encoding green-fluorescent protein (GFP), yellow-fluorescent protein (YFP), cyan-fluorescent protein (CFP), luciferase and BHV-1 tgD were used in this project.

Ovalbumin (OVA) was purchased from Sigma. A truncated secreted version of glycoprotein D (tgD) was produced in transformed Madin Darby bovine kidney cells under regulation of the bovine heat shock 70A (hsp70) gene promoter. The supernatant containing the secreted tgD was collected and purified by size exclusion and ion exchange chromatography. The purified tgD was quantified using a Biorad protein assay and the purity of the protein was established by SDS-polyacrylamide gel electrophoresis.

1.2. Generation of microparticles

1.2.1. PLGA microparticles for DNA vaccines

To develop a microparticle with the desired properties of solubility, size and charge, a number of different PLGA microparticle preparations were generated. Four Resomers were compared, R503S, R504S, R503H, and R502H (Boehringer Ingelheim). In addition, three compounds that add a positive charge necessary for adsorption of negatively charged DNA to the microparticles were tested, dimethyldioctadecylammonium (DDA), polyethylenimine (PEI) and cetyltrimethylammonium bromide (CTAB). These compounds were added at different ratios to the microparticles (Table 1). PVA was used at different concentrations as stabilizer.

All of the preparations were evaluated *in vitro* for solubility and size distribution by a Coulter Multisizer, as well as for DNA adsorption. According to published reports a size of ~ 1-3 μm is optimal (22). Three preparations were selected for further studies *in vitro*, consisting of R503S with 2.5 % PVA and 5 (#3), 15 (#7) or 25 mg DDA (#10). These particles had a homogeneous size distribution (Fig. 1) within the desired size range of 0.5 - 2 μm and acceptable solubility and charge for plasmid DNA loading.

Having tested a number of microparticle preparations, the following procedure was used for the subsequent studies on DNA-coated microparticles. Fifty mg of DDA was dissolved in 1 ml dichloromethane (dcm) and then vortexed, and 100 mg of R503S resomer was dissolved in 2 ml dcm and vortexed. These two suspensions were mixed and vortexed again. After addition of 1 ml TE buffer, the preparation was homogenized for 1 min (setting 4-5). Then 5 ml of 2.5% PVA was added followed by homogenization for 1 min, and addition of another 15 ml of 2.5% PVA. The suspension was stirred for 7 h (setting 6), passed through a 40 μm filter, centrifuged at 10,000 rpm for 10 min and resuspended in 5% sucrose. Finally the suspension was washed three times and resuspended in 20 ml 5% sucrose.

Table 1: Resomer preparations.

Number	Resomer	cation	stabilizer	homogenization
1	R503S	none	1% PVA	1 min
2	R504S	none	5% PVA	1 min/2 min
3	**R503S**	**5 mg DDA**	**2.5% PVA**	**1 min/1 min**
4	R503S	5 mg DDA	5% PVA	1 min/2 min
5	R503S	10 mg DDA	2.5% PVA	1 min/1 min
6	R503S	10 mg DDA	5% PVA	1 min/2 min
7	**R503S**	**15 mg DDA**	**2.5% PVA**	**1 min/1 min**
8	R503H	20 mg DDA	2.5% PVA	1 min/1 min
9	R503S	20 mg DDA	5% PVA	1 min/2 min
10	**R503S**	**25 mg DDA**	**2.5% PVA**	**1 min/1 min**
11	R503S	30 mg DDA	2.5% PVA	1 min/1 min
12	R503S	50 mg DDA	1% PVA	1 min/5 min
13	R503S	100 mg DDA	2% PVA	1 min/2 min
14	R503S	100 mg DDA	5% PVA	1 min/5 min
15	R503S	10 mg CTAB	5% PVA	1 min/2 min
16	R502H	10 mg PEI	0.5% PVA	30 sec/30 sec
17	R502H	10 mg PEI	2.5% PVA	1 min/2 min
18	R502H	20 mg PEI	0.5% PVA	30 sec/30 sec
19	R502H	20 mg PEI	2.5% PVA	1 min/1 min
20	R502H	20 mg PEI	0.5% PVA	1 min/1 min
21	R503H	20 mg PEI	0.5% PVA	30 sec/60 sec
22	R503S	10 mg PEI	0.5% PVA	2 min/1 min
23	R503S	10 mg PEI	2.5% PVA	1 min/1 min
24	R503S	20 mg PEI	0.5% PVA	2 min/1 min
25	R503S	20 mg PEI	0.5% PVA	30 sec/60 sec
26	R503S	20 mg PEI	0.5% PVA	2 min/1 min

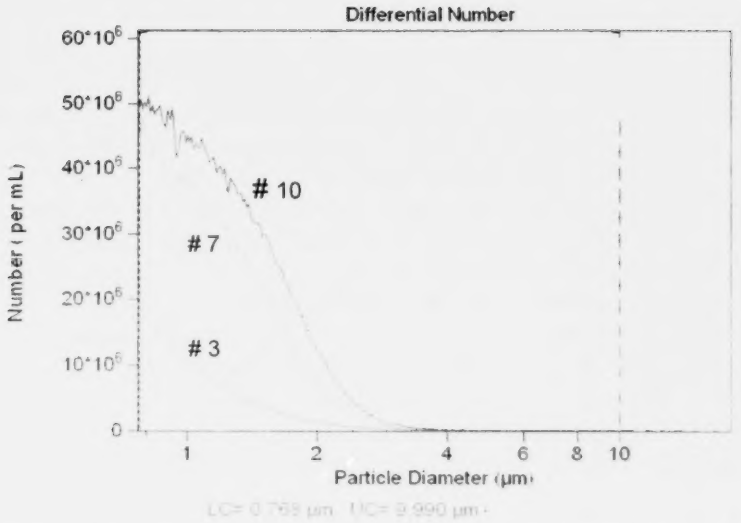
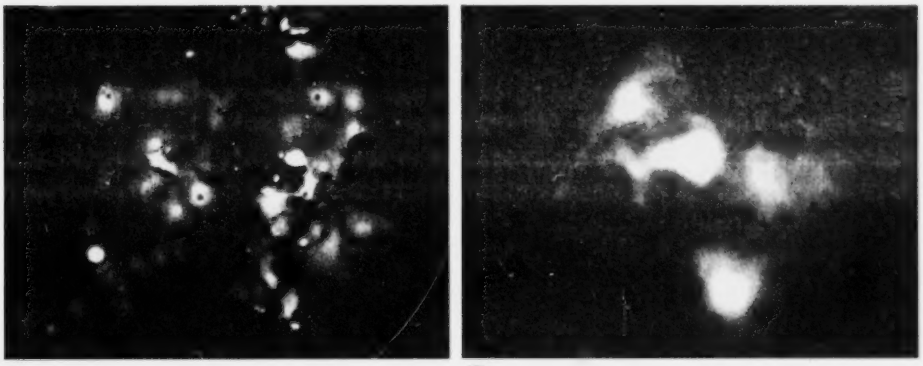


Figure 1: Size distribution of microparticle preparations determined on a Multisizer.

1.2.2. Transfection of COS-7 cells with plasmid DNA-coated PLGA microparticles

To test uptake of plasmid and protein expression, COS-7 cells were incubated with microparticle preparations #3, #7 or #10 coated with plasmid encoding GFP. As shown in Fig. 3 for preparation #7, which was made with R503S and 15 mg DDA, GFP was expressed in the COS-7 cells, confirming that the plasmid-coated microparticles entered the cells and that the plasmid expressed the protein. Interestingly, the kinetics of protein expression observed for commercial transfection reagents was different from that observed for the microparticles. With the commercial reagents we observed protein expression as early as 8 h after transfection and strong expression after 24 h, while with the microparticles we observed very little expression at 24 to 48 h after transfection, followed by a significant increase between days 3 and 6, at which time the cells became too much overgrown to continue the experiment. This confirms the slow release of the plasmid as expected (Fig. 2).



A. **B.**

Figure 2. COS-7 cells transfected with microparticle preparation # 7, R503S with 15 mg DDA, six days after transfection. GFP expression was observed by fluorescence microscopy. A: 10 x; B: 40 x.

1.2.3. PLGA microparticles for protein vaccines

Two sets of microparticles were generated: particles that are used to encapsulate the protein and anionic particles that are used to adsorb the protein. In addition, since our objective is to develop vaccines against BRD, which is caused by pathogens that infect the mucosal surfaces of the respiratory tract, we also generated particles coated with chitosan, to facilitate uptake of vaccine antigen by M cells of the mucosa after intranasal delivery.

In order to adsorb protein to the PLGA microparticles, anionic R504S PLGA particles were created by coating them with SDS or DSS. To save on the expensive target antigen, BHV-1 tgD, we used the academic antigen ovalbumin (OVA) for developmental work. The OVA protein loading efficiency for the SDS-coated particles was 59.6±4.97% (5.96±0.5 μg of OVA/mg of PLGA microparticles (0.59% w/v) and the particle size was 1.98±0.03 μm (anionic PLGA microparticles) and 1.88±0.02 μm (OVA loaded anionic PLGA microparticles). The *in vitro* protein release was ~48% (Fig. 3A). For the DSS-coated particles the OVA protein loading efficiency was 65.14±2.95% (actual protein load: 6.51±0.34 μg of OVA/mg of PLGA microparticles) and the *in vitro* protein release was ~26% (Fig. 3B) after 6 days.

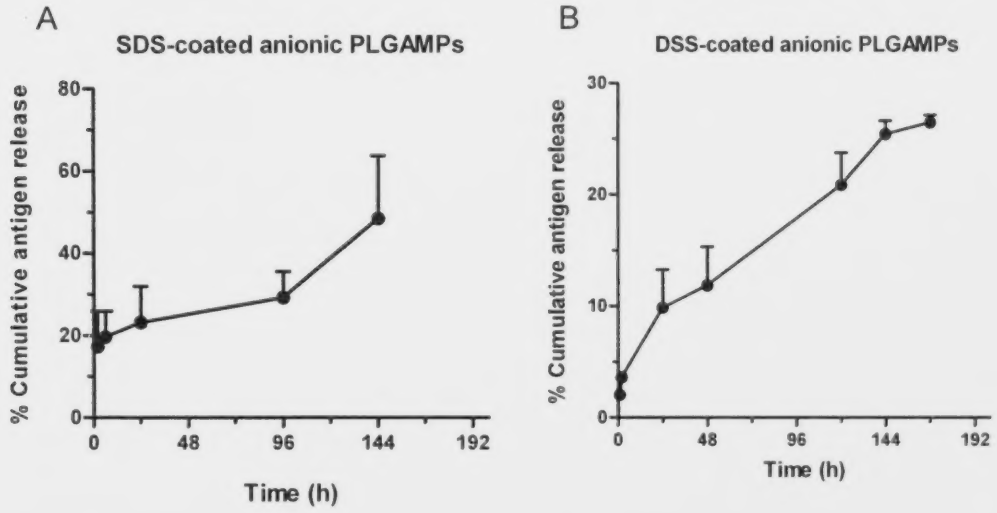


Figure 3. *In vitro* cumulative OVA release from SDS- (A) and DSS- (B) coated anionic R504S PLGA microparticles.

As an approach to incorporate OVA inside the microparticles we complexed OVA with either chitosan or polyphosphazene (PCPP or PCEP) and encapsulated the OVA complexes inside R503H (chitosan) or R504S (polyphosphazene) PLGA microparticles. The protein loading efficiency of the chitosan-OVA-PLGA microparticles was 45.87±.1.47%, and the actual protein load was 45.87±0.14 μg of OVA/mg of PLGA microparticles, whereas the protein loading efficiency of the PCPP-OVA-PLGA microparticles was 38.47±.1.77% and the actual protein load was 96.1±3.64 μg of OVA/mg of PLGA microparticles. The particle size was 2.49±0.17μm. However, the protein release from both of these microparticles was 12%, which was rather low during this time period (Fig. 4).

To evaluate whether these microparticles could be taken up by antigen presenting cells (APCs), murine macrophage cells (RAW 264.7) were incubated with microparticles. At different

time points, the monolayer was washed with sterile PBS and fixed in methanol for 5 min. The cells were stained with Giesma stain (1:20 dilution) and visualized under phase contrast and fluorescence. The percent *in vitro* cell uptake was calculated by selecting three fields in each well and counting the number of cells (400 cells in each field in our experiments) associated with at least one particle. As shown in Fig. 5, the PCPP-OVA-PLGA microparticles were taken up most efficiently, closely followed by DSS-coated anionic microparticles.

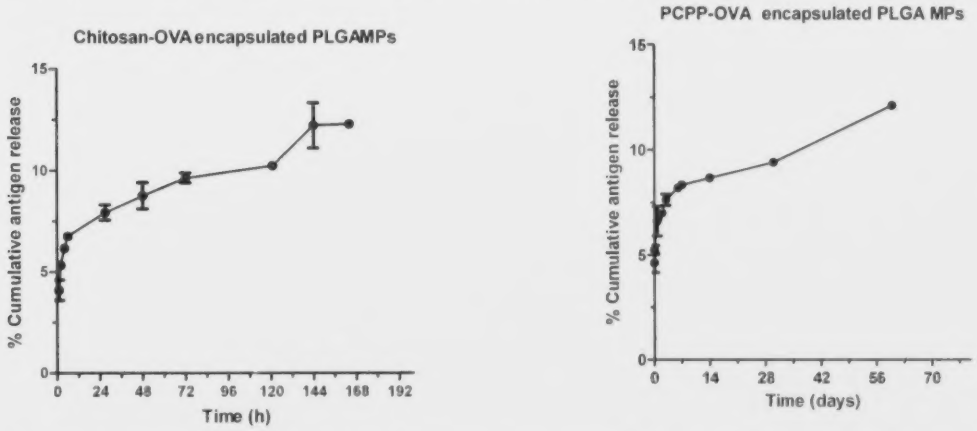


Figure 4. *In vitro* cumulative OVA release from chitosan-OVA encapsulated (A) and PCPP-OVA encapsulated (B) anionic PLGA microparticles.

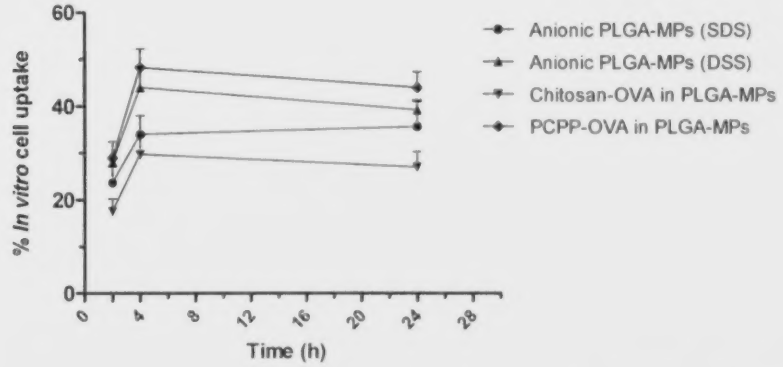


Figure 5. *In vitro* uptake of PLGA microparticles by RAW 264.7 cells.

The next type of PLGA microparticle was based on coating the PCPP-OVA encapsulated R504S PLGA microparticles with chitosan, as chitosan targets the cells of the mucosal tissues. The protein loading efficiency was 44.04±.0.92%, with an actual protein load of 110.27±1.37 ug of OVA/mg of PLGA MPs. The particle size was 0.95±0.01 μm. Release of OVA was 40% after 112 days. The protein release from the chitosan-coated MPS was more efficient than the release from the PCPP-OVA encapsulated particles (Fig. 6).

PCPP-OVA encapsulated and chitosan-modified PCPP-OVA encapsulated PLGA microparticles were made with FITC-labeled OVA to evaluate uptake by APCs (Fig. 7). Uptake of PCPP-OVA encapsulated and chitosan-modified PCPP-OVA encapsulated PLGA microparticles by RAW 264.7 cells was demonstrated (Fig. 8). The chitosan-modified PCPP-

OVA encapsulated PLGA microparticles appeared to be taken up more efficiently. As shown for the chitosan-modified PCPP-OVA encapsulated PLGA microparticles, scanning electron microscopy confirmed the size of the particles, and degradation over a period of 14 days (Fig. 9).

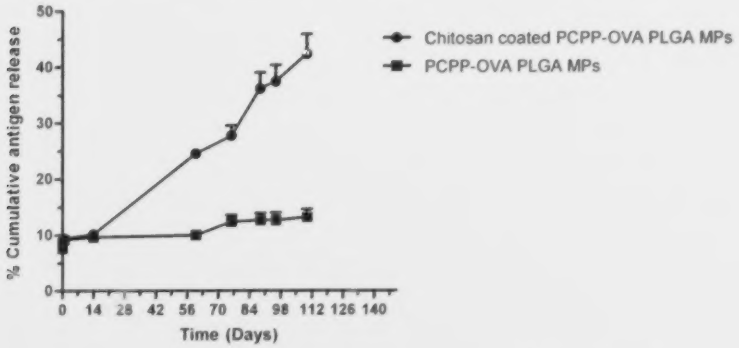


Figure 6. *In vitro* cumulative OVA release from chitosan-OVA and PCPP-OVA encapsulated PLGA microparticles.

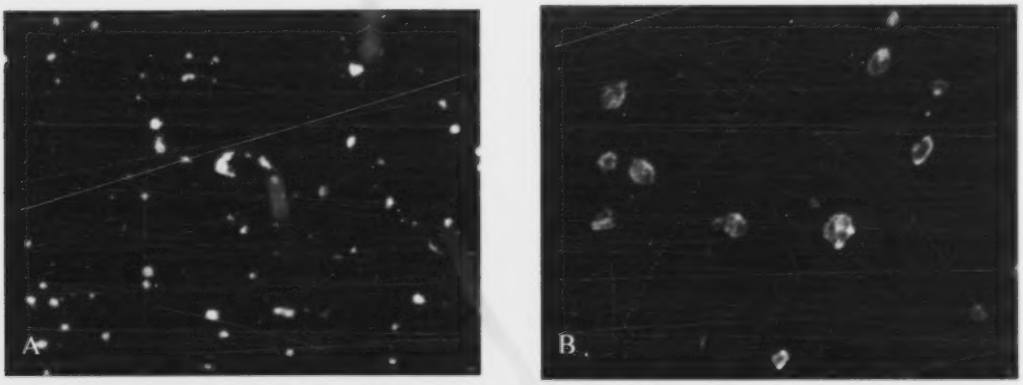


Figure 7. Fluorescent microscopy image of PCPP-FITC BSA loaded PLGA microparticles at 20x (A) and 40x (B) magnification.

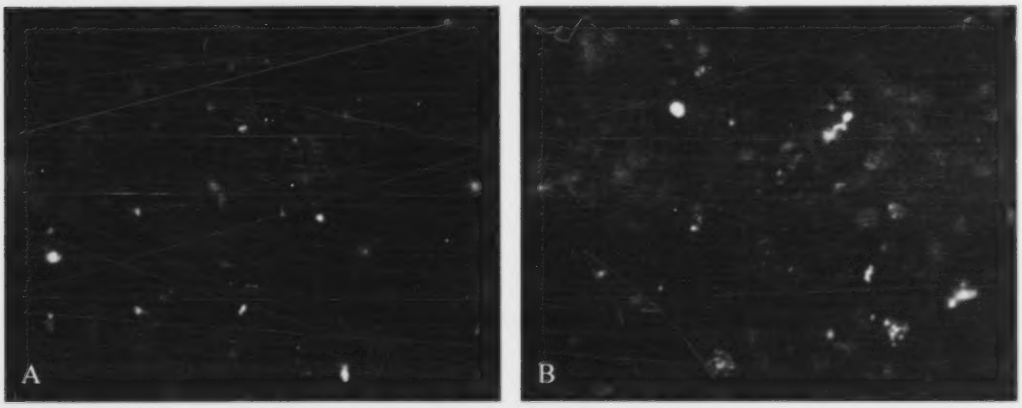


Figure 8. Fluorescent microscopic image of RAW264.7 cells after 4 h incubation with (A) PCPP-OVA encapsulated and (B) Chitosan-modified PCPP-OVA encapsulated PLGA microparticles.

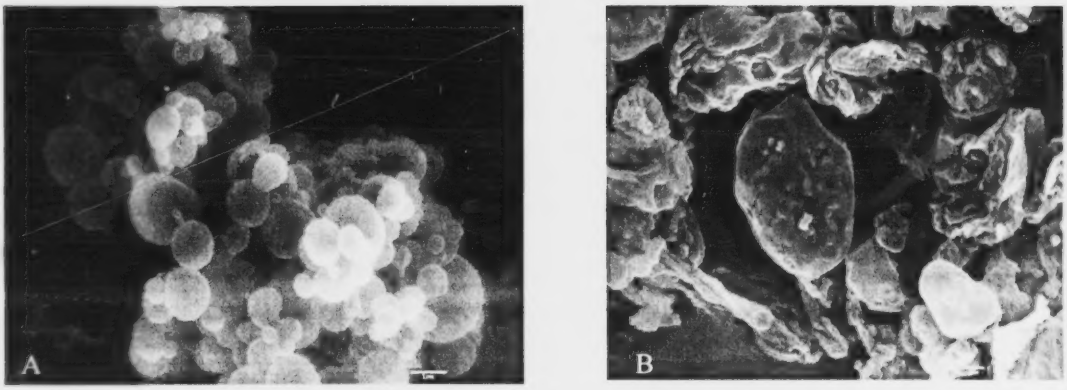


Figure 9. Scanning electron microscopy of chitosan-coated PCPP-OVA encapsulated PLGA microparticles before (A) and 14 days after (B) protein release in PBS (pH 7.4).

2. Assessment of release of plasmid and protein from microparticles in animal tissues.

2.1. Transfection of bovine skin with DNA-coated PLGA microparticles in vivo.

To evaluate the location of protein expressed from microparticles in skin samples, plasmid encoding YFP in saline or coated on R503S-DDA PLGA microparticles was injected into the skin of a calf with a needle-free device, called Biojector. The microparticles had a 20% DNA load. Punch biopsies were taken at 48 h after transfection, sectioned with a cryostat, and analyzed for protein distribution. The distribution and expression of the YFP was very similar after saline and microparticle mediated delivery of plasmid. YFP expression was observed in the top layers of the dermis as well as around the hair follicles (Fig. 10).

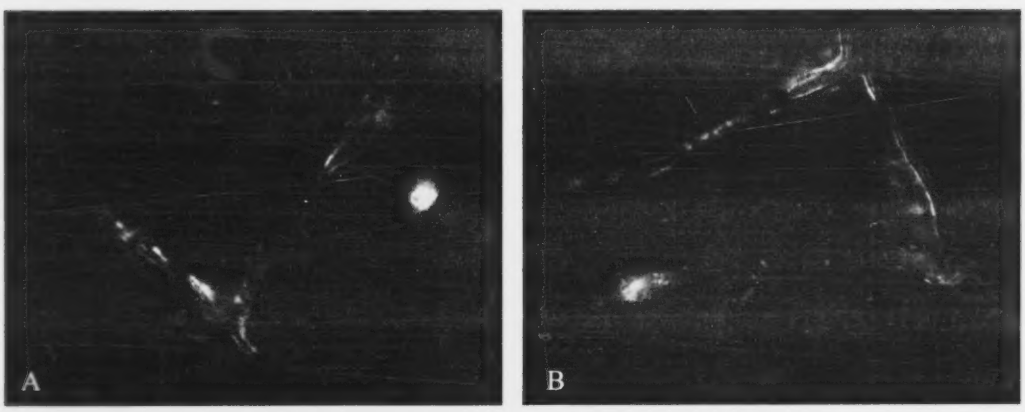


Figure 10. YFP expression in bovine skin 48 h after injection by Biojector with **(A)** YFP plasmid coated microparticle preparation #7, R503S and 15 mg DDA, or **(B)** YFP plasmid in saline.

3. Analysis of distribution of protein produced by microparticle-formulated plasmid in animals.

3.1. Evaluation of protein produced by DNA-coated PLGA microparticles in vivo

To evaluate the amount of protein produced by plasmid DNA-coated microparticles *in vivo*, calves were injected with plasmid encoding luciferase in saline or on R503S-DDA PLGA microparticles. Biopsies were taken at three different time points and homogenized, and the luciferase activity was measured. In contrast to the *in vitro* observations, where the microparticles mediated transfection of plasmid into COS-7 cells, whereas plasmid in saline did not transfect the cells, higher levels of luciferase were detected in the biopsies treated with plasmid in saline than in the biopsies of microparticle-delivered plasmid (Fig. 12). This suggests that we have to further optimize the release characteristics of the microparticles, and that we have to continue to measure this *in vivo*. Alternatively, the plasmid coated microparticles may have been targeted to dendritic cells (DCs) in the skin, which then very quickly moved to the draining lymph nodes, before we took the skin biopsies. If we want to measure this we either have to perform this experiment in mice, or select an injection site in calves or sheep that will drain to lymph nodes that are small enough to be processed and analyzed.

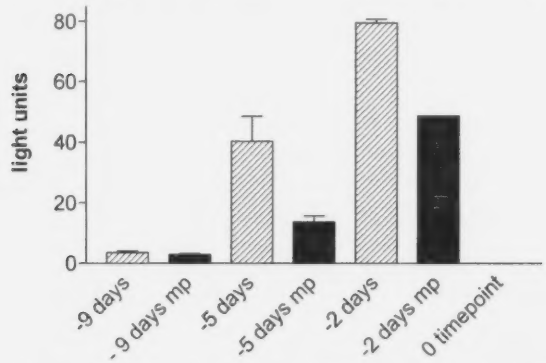


Figure 12. Luciferase expression in bovine skin at 9(-9), 5(-5) and 2(-2) days after injection by Biojector with luciferase-encoding plasmid coated on R503S-DDA PLGA microparticles or in saline.

3.2. Transfection of dendritic cells with PLGA microparticles

Although we originally planned to immediately evaluate the release of plasmid or protein from the microparticles in tissues *in vivo*, we decided to first use mammalian cells in culture to be able to screen several particles in a shorter period of time. We used two systems: COS-7 cells, as described above and DCs, which are the most important antigen presenting cells and critical in induction of a primary immune response.

Since one of the major advantages of microparticles is the fact that they are thought to target DCs, we tested whether our microparticles can mediate transfection of DCs *ex vivo*. Human DCs were exposed to microparticle preparation #7, R503S and 15 mg DDA, and observed over time by light microscopy. Fig. 13A shows adherent DCs with dendrites and non-adherent DCs without dendrites, as well as the microparticles (arrows) around the DCs. After one day the microparticles had disappeared from the cell culture medium and were present inside the DCs (arrows) (Fig. 13B), so they apparently were taken up by the DCs.

Subsequently, since most experimental evidence comes from studies in mice, we performed two experiments to determine whether plasmid-coated microparticles express protein in not only murine APCs but also bovine DCs. Murine splenocytes were isolated and transfected with microparticle preparation #7, R503S and 15 mg DDA, loaded with plasmid encoding CFP to a 2% load. At 24 h after transfection the efficiency of transfection was determined based on CFP production. As shown in Fig. 14A, CFP expression was observed in the murine splenocytes. To further investigate whether microparticle-mediated transfection of plasmid into bovine DCs leads to protein expression, bovine monocyte-derived DCs were transfected with DNA-coated PLGA microparticles combined with either transferrin or polyethylenimine (PEI). The transfection in the presence of PEI did result in expression of YFP protein in some DCs (Fig. 14B), whereas no cells appeared to be transfected in the presence of transferrin.

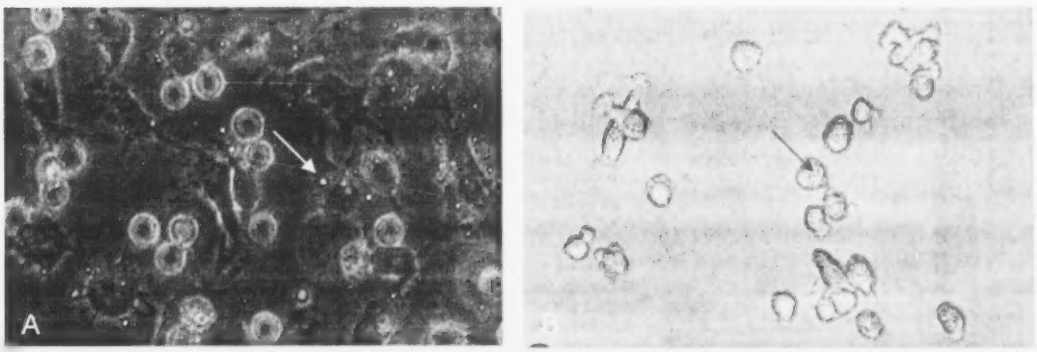


Figure 13. Dendritic cells co-cultured with microparticle preparation #7, R503S and 15 mg DDA, and observed within 2-3 h after adding particles (A) and 24 h later (B).

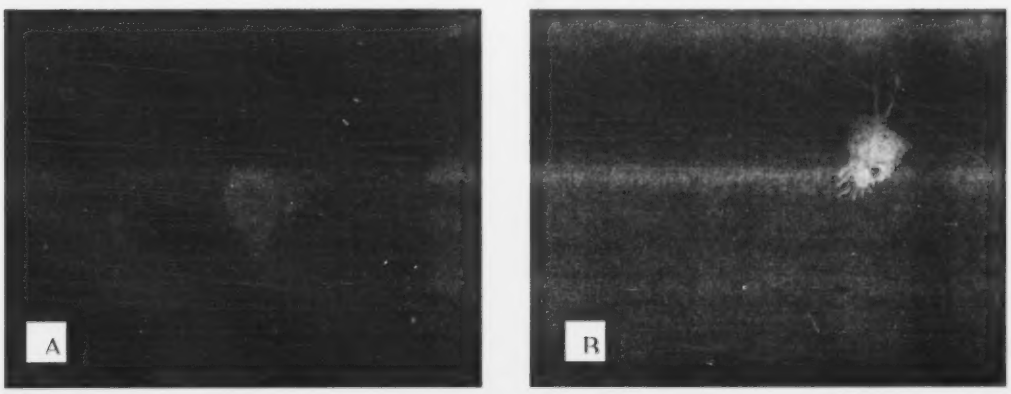


Figure 14. Transfection of antigen-presenting cells by plasmid-coated microparticles. **A.** Murine splenocytes transfected with microparticle made with R503S and PEI, coated with plasmid encoding CFP. **B.** Bovine DCs transfected with preparation #7, R503S and 15 mg DDA, coated with plasmid encoding YFP.

4. Evaluation of immune responses induced by plasmids encoding protective antigen and protein-CpG ODN in microparticles.

We had planned to immunize lambs intradermally with plasmid encoding NaI symporter, and to identify the protein on the basis of iodine accumulation due to the expression of symporter. However, the medical beamline is not yet functional, so these experiments will be performed at a later time, and instead we performed *in vivo* murine trials, as a method to test the microparticles.

4.1. Evaluation of plasmid-coated microparticles *in vivo*

We performed four mouse trials to test the plasmid-coated microparticles. In the first one we compared plasmid in saline to plasmid-coated microparticles at 2, 6 or 12 μg per dose delivered intradermally. This showed no differences between the immune responses induced by the microparticle-mediated delivery and delivery in saline. In the second experiment we delivered intramuscularly, but again there were no differences; in fact, the plasmid in saline outperformed the plasmid-coated microparticles, which suggests that the release of the plasmid from the microparticles may not be optimal *in vivo*. Two more sets of plasmid coated microparticles were evaluated without improvement. Thus, further improvements are needed for the microparticle-DNA formulations.

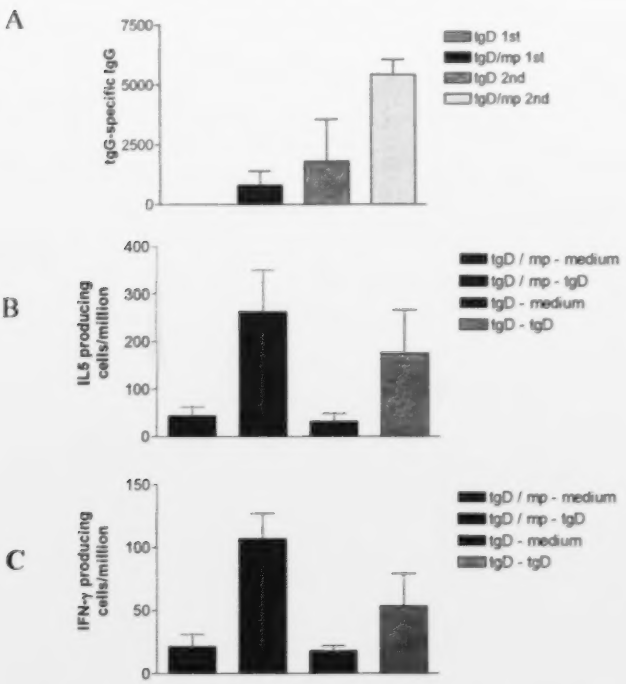


Figure 14. Humoral and cell-mediated immune responses induced in C57BL/6 mice by tgD protein delivered in saline (tgD) or on R503S PLGA microparticles (tgD/mp). **(A)** tgD-specific IgG titer after the 1st and 2nd immunization; **(B)** tgD-induced induced IL-5 secreting cells per 1×10^6 splenocytes after *in vitro* re-stimulation with medium or tgD; **(C)** tgD-induced induced IFN-γ secreting cells per 1×10^6 splenocytes after *in vitro* re-stimulation with medium or tgD.

4.2. Evaluation of protein-coated microparticles in mice *in vivo*

We decided to use the mouse model for evaluation of the protein-coated microparticles before vaccinating sheep or cattle. In the first trial mice were vaccinated subcutaneously (SC) with a protective antigen of BHV-1, 1 μg tgD, in 20 μl either in PBS or coated on 1 mg R503S PLGA microparticles (2% loading). The IgG titers induced by the microparticle formulation were higher than the titers elicited by tgD in PBS. Furthermore the numbers of IL-5 and IFN-γ secreting T cells tended to be also larger in the microparticle-vaccinated group of mice (Fig. 14).

In the second trial the microparticle formulation was compared to the strongest adjuvant we have for tgD in mice, the protein load was varied, and the immune responses were followed for a longer period of time after one vaccination. Mice were vaccinated with 1 μg tgD either in PBS without adjuvant, or with a strong oil-based adjuvant and immune stimulator, 10% Emulsigen and 10 μg CpG ODN (EM/CpG), or in microparticles at a 2%, 4% or 8% load. The mice were re-vaccinated on day 84 and the immune response was further evaluated.

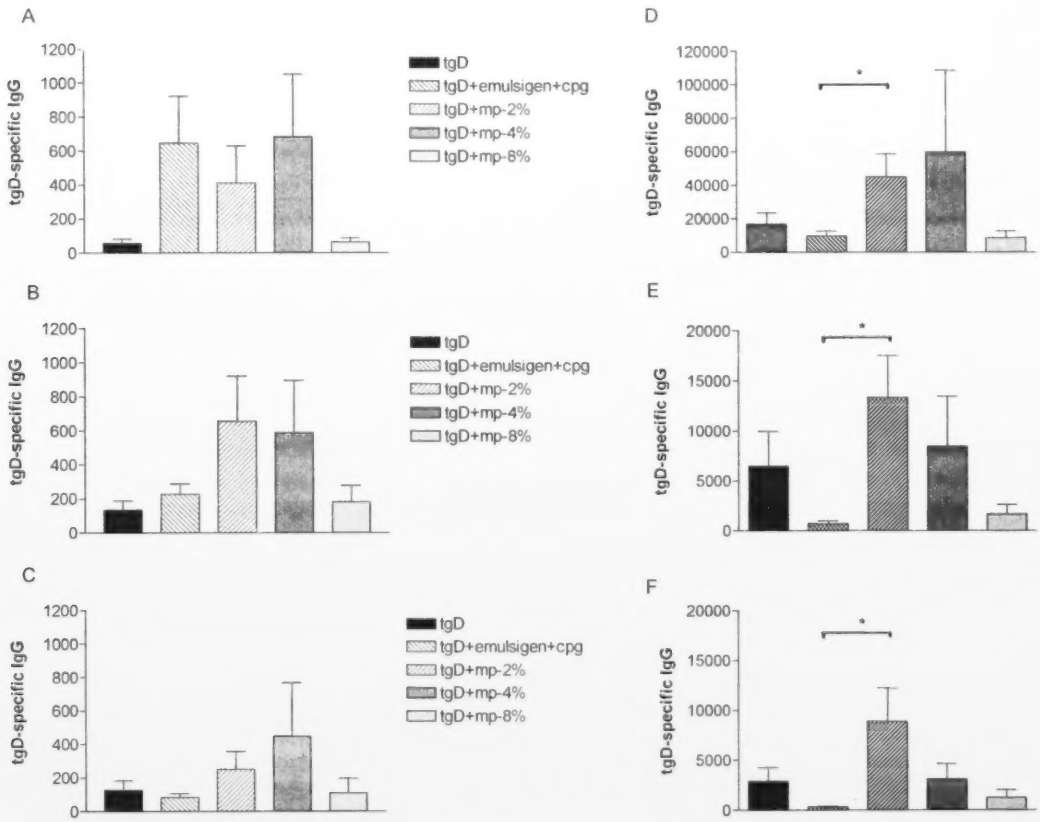


Figure 15. Humoral immune responses induced in C57BL/6 mice by tgD protein delivered in saline (tgD), formulated with 10% Emulsigen and 10 μg CpG ODN (tgD+emulsigen+cpg) or on R503S PLGA microparticles at a loading of 2%, 4% or 8%. (tgD+mp). tgD-specific IgG titer 14 days **(A),** 28 days **(B),** and 56 days **(C)** after the first immunization on day 0, and 10 days **(D)**, 38 days **(E)** and 66 days **(F)** after the second immunization on day 84. Significance of differences: * $p < 0.05$.

This experiment showed very low immune responses to tgD in PBS, a strong, but transient response to tgD+Emulsigen+CpG, and a strong and longer lasting response to tgD in microparticles at 2% or 4% loading, whereas at 8% loading the responses were equivalent to the responses to tgD without adjuvant, in PBS (Fig 15).

In addition to the PLGA microparticles, the DSS-coated anionic PLGA microparticles, chitosan-OVA encapsulated PLGA microparticles and PCPP-OVA encapsulated PLGA microparticles were evaluated in mice using 10 or 25 μg OVA and a SC route. In addition, the chitosan-coated PCPP-OVA PLGA microparticles were tested at 25 μg intranasally, as chitosan is known to target mucosal tissues. As shown in Fig. 16, the DSS-coated anionic microparticles induced the strongest immune responses SC, whereas the chitosan-coated PCPP-OVA PLGA microparticles induced the highest serum IgG titers when delivered intranasally. Furthermore, intranasal delivery resulted in mucosal IgG in the nasal fluids (Fig. 17).

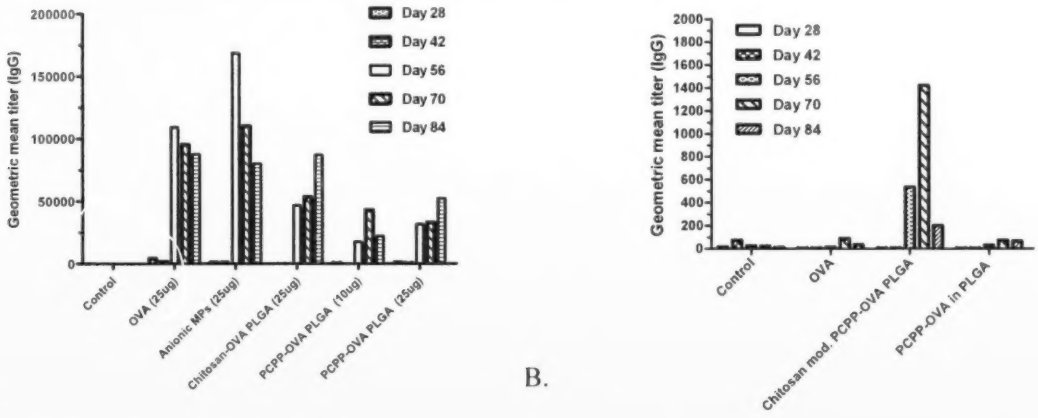


Figure 16. Humoral immune responses induced in C57BL/6 mice by OVA protein delivered in PBS (OVA), OVA formulated in DSS-coated anionic R504S PLGA microparticles, chitosan-OVA encapsulated PLGA microparticles or PCPP-OVA encapsulated PLGA microparticles delivered SC **(A)** or by OVA in PBS, PCPP-OVA encapsulated PLGA microparticles or chitosan-coated PCPP-OVA PLGA microparticles delivered intranasally **(B)**.

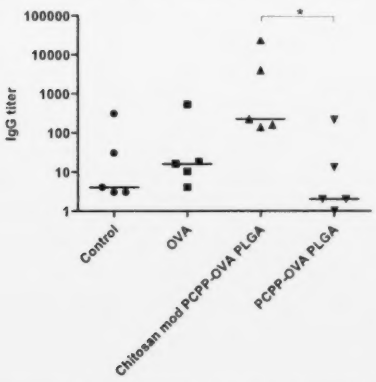
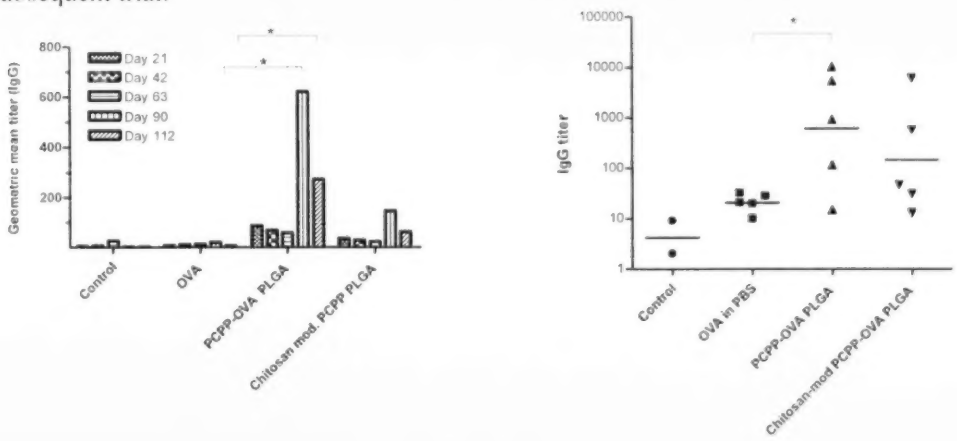


Figure 17. Mucosal IgG in the nasal fluids induced in C57BL/6 mice by OVA protein delivered in PBS (OVA), in PCPP-OVA encapsulated PLGA microparticles or in chitosan-coated PCPP-OVA PLGA microparticles intranasally and measured on day 70. Significance of differences: * $p < 0.05$.

The PCPP-OVA encapsulated and chitosan-modified PCPP-OVA PLGA microparticles were also evaluated in sheep using a SC delivery route. The PCPP-OVA encapsulated PLGA microparticle formulations induced higher IgG titers than OVA in PBS ($p < 0.05$) (Fig. 18).

Overall, these results demonstrated that in mice the chitosan-modified PCPP-OVA microparticles can be used for intranasal delivery of antigen. In sheep the PCPP-OVA PLGA microparticles induced stronger responses than OVA alone, when delivered SC; we expected these microparticles to be efficient in mice as well, but since the OVA in PBS induces very high antibody responses, we may need to reduce the dose to detect a difference, which we did in the subsequent trial.



A B

Figure 18. Humoral immune responses induced in sheep by OVA protein delivered in PBS (OVA), or formulated in PCPP-OVA PLGA microparticles or in chitosan-coated PCPP-OVA PLGA microparticles delivered SC (A) Geometric mean IgG titers; (B) IgG titers of individual animals and median titers on day 90. Significance of differences: * $p < 0.05$.

4.3. Evaluation of protein-coated microparticles and CpG microparticles in mice *in vivo*

The subsequent trials were designed to evaluate the effect of co-formulation of antigens with the immunostimulatory molecule, CpG ODN, in PLGA microparticles. Two proteins, OVA and tgD, were used for these trials.

OVA was formulated at 10 μg with two different polyphosphazenes, PCPP or PCEP, inside PLGA microparticles as described above, but lower doses of OVA were used, as the previously used doses were thought to be too high to observe effects of microparticle formulation. In addition, PCEP-OVA PLGA and PCPP-OVA PLGA microparticles were co-formulated with chitosan-CpG-PLGA microparticles. The mice were immunized SC. As shown in Fig. 19, the groups immunized with PCPP-OVA+CpG and PCEP-OVA+CpG developed significantly higher titers ($p < 0.01$ and $p < 001$ respectively) in comparison to the placebo group. PCPP-OVA and PCEP-OVA PLGA microparticles induced equivalent IgG titers; furthermore, the addition of Chit-CpG to the PCEP OVA formulation significantly enhanced the IgG titers ($p < 0.05$). In addition, cell-mediated immune responses were determined for selected groups. The numbers of IFN-γ secreting cells were significantly higher in the PCEP-OVA and PCEP-OVA/Chit-CpG groups than in the PBS group ($p < 0.001$ and $p < 0.01$ respectively),

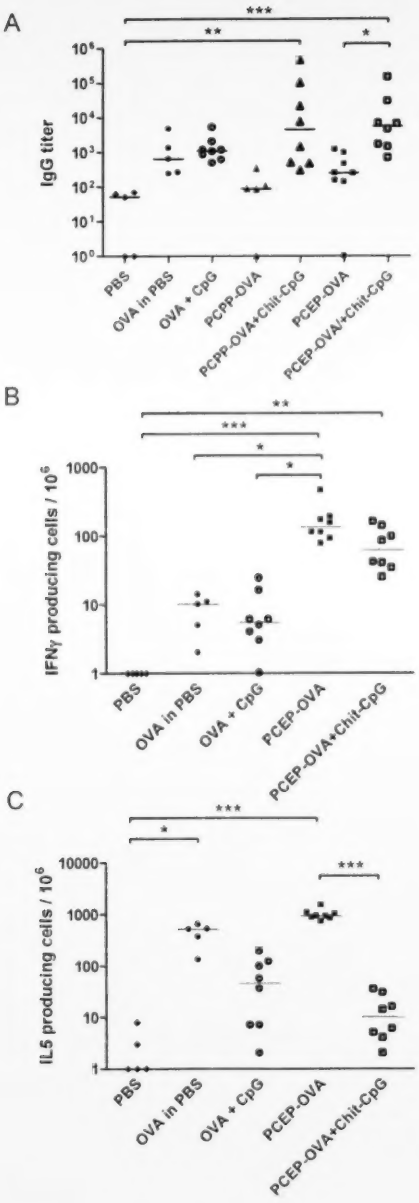


Figure 19. Humoral and cell-mediated immune responses induced in C57BL/6 mice by OVA protein delivered in PBS, with CpG ODN, or formulated with PCPP or PCEP in PLGA in the presence or absence of CpG ODN in Chitosan-PLGA. **(A)** OVA-specific IgG titer after the 2nd immunization; **(B)** OVA-induced induced IFN-γ secreting cells per 1×10^6 splenocytes after in vitro re-stimulation with OVA; **(C)** OVA-induced induced IL-5 secreting cells per 1×10^6 splenocytes after in vitro re-stimulation with OVA. Significance of differences: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

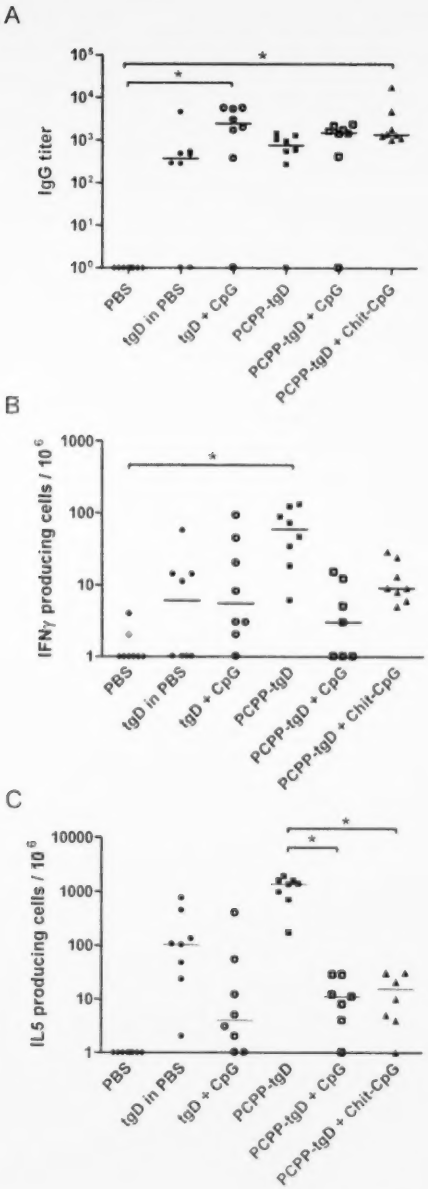


Figure 20. Humoral and cell-mediated immune responses induced in C57BL/6 mice by BHV-1 tgD protein delivered in PBS, with CpG ODN, or formulated with PCPP in PLGA in the presence of CpG ODN or CpG ODN in Chitosan. **(A)** tgD-specific IgG titer after the 2nd immunization; **(B)** tgD-induced induced IFN-γ secreting cells per 1×10^5 splenocytes after in vitro re-stimulation with medium or tgD; **(C)** tgD-induced induced IL-5 secreting cells per 1×10^6 splenocytes after in vitro re-stimulation with medium or tgD. Significance of differences: * $p < 0.05$.

whereas the PCEP-OVA group developed more IFN-γ secreting cells than the OVA in PBS and OVA-CpG groups ($p < 0.05$). The OVA in PBS ($p < 0.05$) and PCEP-OVA ($p < 0.001$) immunized mice had significantly more IL-5 secreting cells than the placebo group. The addition of CpG ODN to OVA+PBS and PCEPP-OVA resulted in a reduction in IL-5 secreting cells, which was highly significant for PCEP-OVA ($p < 0.001$). In the case of OVA in PBS, this resulted in a shift towards a more balanced immune response, whereas the response induced by PCEP-OVA/Chit-CpG was Th1 biased.

In order to evaluate whether these polyphosphazene-CpG formulations would be usable for the antigen of interest, BHV-1 tgD, we formulated tgD at 1 μg with PCPP inside PLGA microparticles as described above. In addition, PCPP-OVA PLGA microparticles were co-formulated with CpG ODN or Chitosan-CpG-PLGA microparticles. Formulation of tgD in PCPP-PLGA particles tended to enhance IgG titers and numbers of IFN-γ and IL-5 secreting cells (Fig. 20); however, due to the variation between animals there was no significant difference. Furthermore, the PCPP-tgD group was the only one with significantly enhanced numbers of IFN-γ secreting cells when compared to placebo ($p < 0.05$). Addition of CpG ODN or Chitosan-CpG-PLGA microparticles to the respective formulations tended to enhance IgG titers, whereas the numbers of IL-5 secreting cells were decreased ($p < 0.05$), suggesting a shift to a more balanced immune response.

Overall, these results demonstrate that complexation of protein with PCPP or PCEP and incorporation into PLGA microparticles results in enhancement of cell-mediated immune responses, while there is little effect on the antibody responses; adsorption of CpG ODN to Chitosan PLGA microparticles lead to enhanced and/or more consistent cell-mediated immune responses.

4.4. Evaluation of protein-coated microparticles and CpG microparticles in cattle *in vivo*.

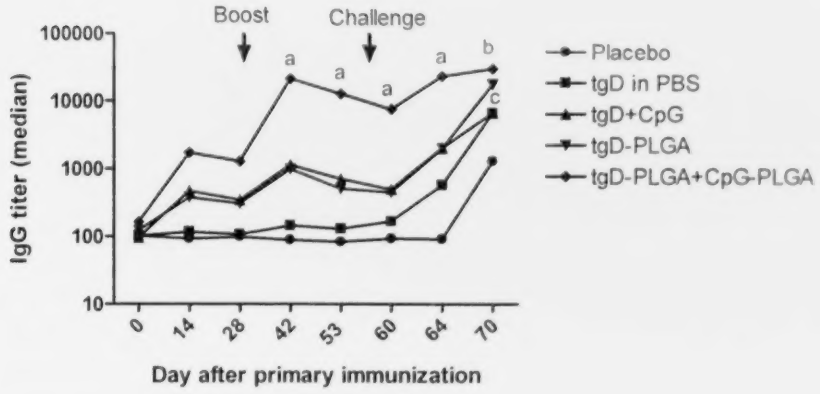
To evaluate whether the formulation of BHV-1 tgD protein and/or CpG ODN in microparticles would result in enhancement of immune responses and protection in cattle, thirty 6- to 7-months-old Hereford calves were randomly allocated to one of five treatment groups of six animals each. They were immunized twice with a four-week interval in the neck with one of the following formulations: 1) PBS, 2) tgD in PBS; 3) tgD in PBS with 400 μg ODN 2007; 4) tgD adsorbed to PLGA microparticles; or 5) tgD and 400 μg ODN 2007, both adsorbed to PLGA microparticles. The tgD was formulated in 2 ml at 50 μg/dose and delivered SC.

Two weeks after the last immunization, the calves were each exposed for 4 min to an aerosol of 10^7 pfu per ml of BHV-1 strain 108, which was generated by a Devilbis Nebulizer, model 65. On the day of challenge and for 12 days afterwards, the calves were clinically assessed by a veterinarian unaware of the experimental status of the animals, and the temperatures and weights were measured. Sera were collected before each vaccination, as well as prior to and two weeks after BHV-1 challenge. Peripheral blood was collected before and after the final boost as well as two weeks after BHV-1 challenge. Nasal swabs were used to obtain nasal fluids from all animals three days before challenge, as well as on days 2, 4, 6, 8 and 10 post-challenge. This trial was carried out according to the guidelines provided by the Canadian Council for Animal Care.

Based on serum antibody titers shown in Figure 21 the group vaccinated with tgD-PLGA+CpG PLGA microparticles developed a significantly stronger response compared to all other groups after two vaccinations, as well as after challenge, whereas 14 days after challenge

the tgD-PLGA+CpG-PLGA vaccinated group had higher titers than the Placebo, tgD and tgD+CpG groups. The calves vaccinated with tgD+PLGA had a higher titer than the animals in all but the tgD-PLGA+CpG-PLGA groups 14 days after challenge. After two immunizations the tgD-PLGA+CpG-PLGA group developed higher virus neutralizing antibody titers than the placebo, tgD in PBS and tgD PLGA groups ($p < 0.05$), whereas after challenge tgD-PLGA+CpG-PLGA group had significantly higher titers than all other groups ($p < 0.001$).

A



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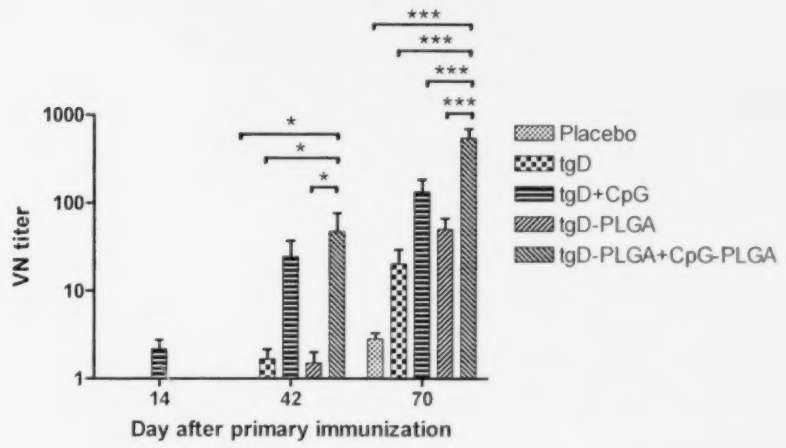

Figure 21. Humoral immune responses after vaccination with BHV-1 tgD. Calves were immunized twice with. 1) PBS (placebo), 2) tgD in PBS; 3) tgD in PBS with 400 µg ODN 2007; 4) tgD adsorbed to PLGA microparticles; or 5) tgD and 400 µg ODN 2007, both adsorbed to PLGA microparticles. Two weeks after the last immunization the calves were challenged intranasally with BHV-1. **(A)** Median ELISA titers. Significance of differences: a: difference between tgD-PLGA+CpG-PLGA and all other groups; b: difference between tgD-PLGA+CpG-PLGA and the Placebo, tgD, tgD+CpG groups; c: difference between the tgD-PLGA and Placebo, tgD and tgD+CpG groups. **(B)** Mean virus neutralization titers. Significance of differences: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Cell-mediated immune responses were evaluated based on the activation of IFN-γ secreting T cells. The tgD-PLGA+CpG-PLGA group developed significantly more IFN-γ secreting cells in the PBMCs than the Placebo and tgD in PBS groups after the second vaccination ($p < 0.05$), and also in comparison with the tgD+CpG group after challenge ($p < 0.05$) (Fig. 22).

The tgD-PLGA+CpG-PLGA group also lost less weight and did have lower temperatures than all other groups, as shown in Figure 23. However, there were no differences between the other groups. Finally, the tgD-PLGA+CpG-PLGA group shed less virus than the other groups confirming that this formulation improves the protective efficacy of the subunit tgD vaccine (Fig. 24).

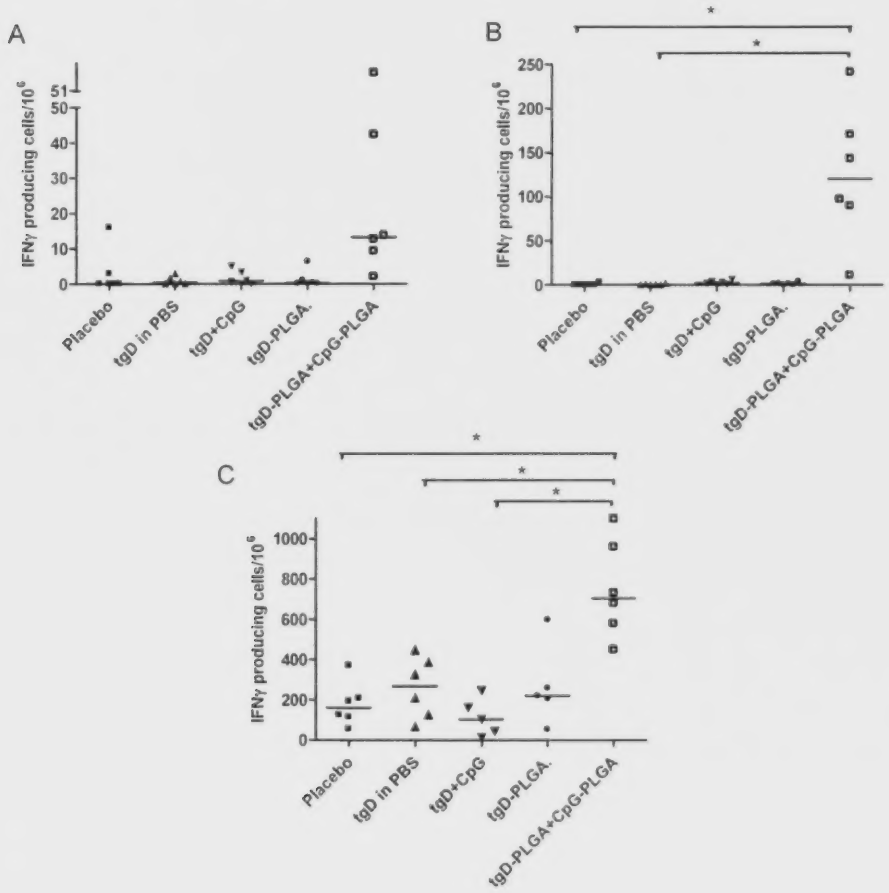

Figure 22. Cell-mediated immune responses after vaccination with BHV-1 tgD. Calves were immunized twice with. 1) PBS (placebo), 2) tgD in PBS; 3) tgD in PBS with 400 μg ODN 2007; 4) tgD adsorbed to PLGA microparticles; or 5) tgD and 400 μg ODN 2007, both adsorbed to PLGA microparticles. Two weeks after the last immunization the calves were challenged intranasally with BHV-1. IFN-γ secreting cells were determined in the PBMCs after the 1st **(A)** and 2nd **(B)** immunization, as well as after BHV-1 challenge **(C)**. Significance of differences: * $p < 0.05$.

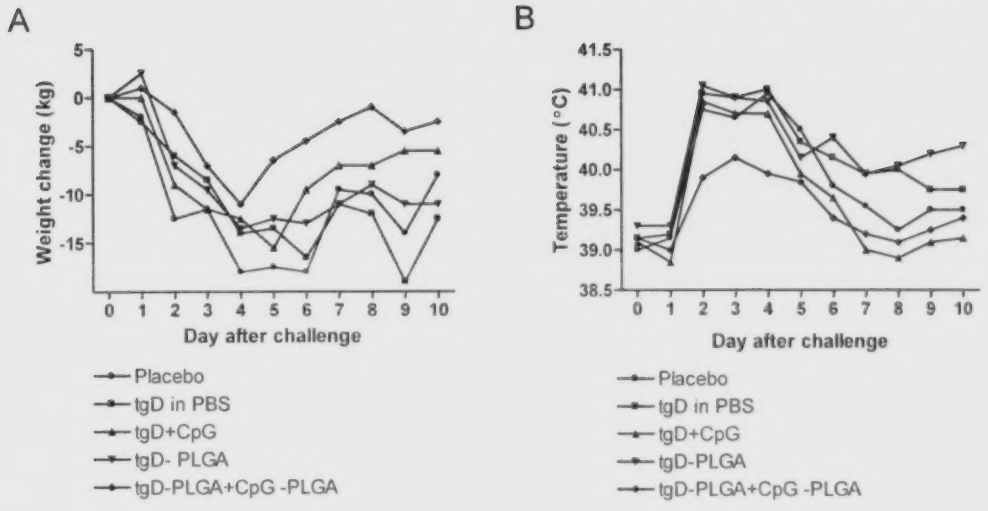

Figure 23. Clinical course after challenge with BHV-1. Calves were immunized twice with 1) PBS (placebo), 2) tgD in PBS; 3) tgD in PBS with 400 µg ODN 2007; 4) tgD adsorbed to PLGA microparticles; or 5) tgD and 400 µg ODN 2007, both adsorbed to PLGA microparticles. Two weeks after the last immunization the calves were challenged intranasally with BHV-1. **(A)** Cumulative weight change (median). **(B)** Average temperatures (median).

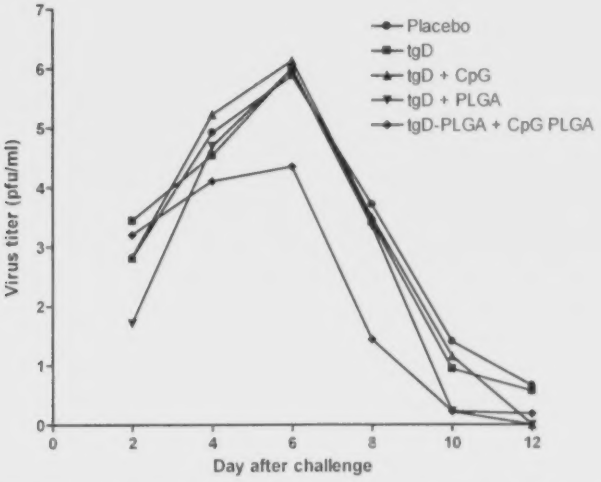

Figure 24. Virus shedding after challenge with BHV-1. Calves were immunized twice with 1) PBS (placebo), 2) tgD in PBS; 3) tgD in PBS with 400 µg ODN 2007; 4) tgD adsorbed to PLGA microparticles; or 5) tgD and 400 µg ODN 2007, both adsorbed to PLGA microparticles. Two weeks after the last immunization the calves were challenged intranasally with BHV-1. Viral titers are expressed as geometric means.

CONCLUSIONS AND RECOMMENDATIONS

Several protein microparticles were promising. One of the protein-coated PLGA microparticles induced prolonged immune responses when delivered subcutaneously. Incorporation of protein in polyphosphazene-PLGA microparticles also resulted in stronger and more balanced immune responses in mice and enhanced immune responses in sheep. Polyphosphazene-PLGA microparticles coated with chitosan, which targets to the mucosal surfaces, induced strong immune responses when delivered intranasally to mice. This is of importance for the ability to deliver antigens mucosally, which is expected to lead to improved protection from respiratory disease. Finally, microparticles proved to be a good vehicle for delivery of the immune modulator CpG ODN, which has strong adjuvant activity, both in mice and in cattle.

With these results we have established microparticle formulations that are promising for delivery of protein and CpG ODN, so future studies will involve optimizing the protein-CpG ODN microparticle formulations for viruses involved in bovine respiratory disease, in particular IBR and BVDV.

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